MicroBioTest Protocol

AOAC USE DILUTION TEST
BROAD SPECTRUM

Prepared for:

DISHWASHER MAGIC LLC.
P.O. Box 61
Wyckoff, NJ 07481

Sponsor's representative

WAGNER REGULATORY ASSOCIATES
2314 Andys Lane
Wilmington, DE 19810

March 20, 2001

OBJECTIVE:

This test is designed to substantiate disinfectant effectiveness claims for a product to be registered with the Environmental Protection Agency. It measures the potential of the test material to disinfect hard surfaces contaminated with bacteria. The test follows *Official Methods of Analysis*, Fifteenth edition, 1990, AOAC; is required by EPA DIS/TSS 1 & 2; and will be conducted under EPA GLP regulations (40 CFR §160).

TESTING CONDITIONS:

A total of sixty replicates per microorganism per lot of test material will be evaluated using three lots of the test material, one of which is at least sixty days old. *Staphylococcus aureus* and *Salmonella choleraesuis* cultures dried on stainless steel penicylinders will be exposed to the test material at the temperature and for the time stipulated by the sponsor. The carriers will be removed from the test solution, neutralized and cultured.

MATERIALS

A. Test agents supplied by the sponsor: see last page.

The test substance is tested as supplied by the sponsor unless directed otherwise. All operations performed on the test substance such as dilution or specialized storage conditions must by specified by the sponsor prior to the initiation of testing.

The sponsor assures MicroBioTest, Inc. testing facility management that the test substance has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

MicroBioTest will retain all unused test materials for a period of three months after completion of the test, then discard them in a manner that meets the approval of the safety officer.

B. Materials supplied by MicroBioTest, Inc., including, but not limited to:

1. Challenge microorganisms, required by EPA DIS/TSS 1:

   a. *Staphylococcus aureus*, ATCC 6538
   b. *Salmonella choleraesuis*, ATCC 10708

2. Media and reagents:

   a. Nutrient Agar plates (NA)
   b. Nutrient Broth (NB)
   c. Asparagine solution, 0.1%
   d. Sodium hydroxide solution, 1N (NaOH)
   e. Recovery Broth (NB or Fluid Thioglycollate Medium)
   f. Recovery broth with neutralizer
   g. Deionized water (DI)
   h. PBS with 1% Polysorbate 80
   i. Heat-inactivated horse serum (if required)

3. Laboratory equipment and supplies including polished stainless steel penicylinders

EXPERIMENTAL DESIGN:

A. Inocula preparation:

Bacteria from stock cultures will be transferred into NB using a 4-mm loop and incubated at 37±2C. Daily transfers will be made for at least three consecutive days (but no more than 30). Tubes of 10-mL NB will be inoculated with one loopful of inoculum per tube and incubated at 37±2C. After 48-54 hours, cultures will be used for contaminating the carriers. For each microorganism, the NB cultures will be pooled into a sterile flask.

Each inoculum will be agitated on a Vortex-type mixer for 3-4 seconds. The inoculum will be allowed to set for ten minutes then decanted into a sterile flask, leaving all residues in the original flask. Twenty-mL aliquots will be transferred into sterile tubes with mixing of the inoculum between transfers.

B. Carrier preparation:

The carriers will be soaked overnight in 1N NaOH, rinsed with tap water until a neutral pH is reached, then rinsed twice with DI. Cleaned carriers will be placed in multiples of 10 into tubes, covered with 0.1% asparagine solution, steam-sterilized for 20 min at 121C, cooled and stored at room temperature until use.

The carriers will be placed into the broth cultures and remain in contact with the inocula (20 carriers per tube of 20-mL inoculum) for 15 min at ambient temperature; then they will be removed from the broth and placed into sterile, Petri dishes matted with filter paper, and dried at 37±2C for 40 min.

C. Test material preparation:

The disinfectant will be prepared according to the sponsor's specifications and dispensed in 10-mL aliquots into sterile 25x150 mm test tubes. The tubes will be placed in a water bath and allowed to come to test temperature for at least ten minutes before testing.

D. Test:

Tubes containing the test material will be maintained at the temperature specified by the sponsor throughout the test. One contaminated carrier will be added to each tube; the tube swirled to mix; and the carrier allowed to remain in contact with the test agent for a time specified by the sponsor of the study. After the exposure time, the carriers will be removed, transferred to neutralizing broth and the tubes will be thoroughly shaken. All tubes will be incubated at 37±2C for 48±2 hr and the results recorded as visible growth (+) or no visible growth (–).

E. Controls:

1. Neutralizer effectiveness:

Three tubes containing ten mL of one of the test material lots will be allowed to equilibrate to 20±1C for at least 10 min. A single sterile carrier will be added to each tube and held for the same time as the test carriers. After the exposure time, each carrier will be added to a tube containing recovery broth with neutralizers and fewer than 100 CFU of the challenge microorganism will be added to each tube. The CFU added to each tube will be confirmed.

This procedure will be repeated for each challenge microorganism. All tubes and plates will be incubated with the test.

2. Carrier counts:

If serum is added to the inocula, the average CFU per carrier will be determined for each microorganism on three carriers. Dried carriers will be placed individually into tubes containing 10 mL PBS + 1% Polysorbate 80. The tubes will be subjected to ultrasound for 5 min in a cleaning (not cavitating) sonicator. Serial ten-fold dilutions of each suspension will be performed in PBS blanks. Duplicate one-mL aliquots from selected dilutions will be plated in Nutrient Agar pour plates. All plates will be incubated with the test and the average CFU/carrier determined.

3. Viability controls:

Two inoculated carriers for each challenge microorganism will be inoculated into tubes of recovery broth with neutralizers and incubated with the test to serve as comparison for the test cultures.

4. Bacteriostasis control:

   If, after two days incubation, no growth is observed in any tube for one challenge microorganism, all tubes will be streaked onto TSA and incubated for 24±2 hr at 37±2C. No growth on these plates will negate bacteriostasis as the cause for lack of growth in the test tubes.

5. Sterility controls:

   One tube of recovery broth with neutralizers containing a single sterile carrier and one tube of LB will be incubated with the test.

6. Confirmation of challenge microorganisms:

   All of the viability controls and at least 20% of the test tubes showing growth will be streaked onto Tryptic Soy Agar plates and incubated for 24±2 hours at 37±2C. Gram stains will be performed from these streaks in order to confirm growth of the challenge microorganisms.

PRODUCT EVALUATION CRITERIA:

According to EPA, the compound passes the test if visible growth is observed in no more than one of the subculture broths (1/60) for any microorganism for any lot of test material and the controls meet the stipulated resistance criteria.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance.

- The carrier counts should be at least 10^4 CFU/carrier.

STUDY DATES:

The anticipated date of study initiation (date when the study director signs the protocol) is upon receipt of test material and letter of authorization with a purchase order number and a signed protocol. The date for submission of the final report to the sponsor will be within one month of laboratory phase completion. The date the study director signs the final report is the study completion date.

DATA PRESENTATION:

The final report will include the following information:

- The number of positive carriers per microorganism per lot.
- The average colony-forming units per carrier

REPORT FORMAT:

MicroBioTest employs a standard report format for each test design. Each final report provides the following information:

- Sponsor identification and test material identification
- Type of test and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results
- Methods and evaluation criteria
- Signed Quality Assurance and Compliance Statements

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test material records, final report, and correspondence relevant to this study, between MicroBioTest and the sponsor will be stored in the archives at MicroBioTest, Inc., 105B Carpenter Drive, Sterling, VA 20164.

All changes or revisions to the approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of the change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test agent; and the type of neutralizer(s) to be employed in the test will be addressed in a project sheet issued separately for each study.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request. This study will be conducted at MicroBioTest, Inc., 105B Carpenter Drive, Sterling, VA 20164.

MISCELLANEOUS INFORMATION:

The following information is to be completed by sponsor's representative:

A. Name and address: DISHWASHER MAGIC LLC.
P.O. Box 61
Wyckoff, NJ 07481

B. Test agent: Dishwasher Magic (12fL.oz./bottle)

1st Lot No: 3-12012-1 2nd Lot No: 12-12012-0

3rd Lot No (≥60 days old): 9-12012-0

Active ingredient: citric Acid

Dilution to be tested: one 12-fl oz bottle diluted in 7 quarts Diluent: water @400ppm hardness

Exposure time: 12 min Exposure temperature: 51 ±1C.

C. Organic load: ☐ 5% serum (added to inoculum)

D. Precautionary/storage conditions: see MSDS and Certificate of Analysis

REPORT HANDLING:

This information is to be: ☑ submitted to the EPA ☐ submitted to the FDA
☐ used for internal purposes only
☐ Other: _____

PROTOCOL APPROVAL:

Sponsor: James M Wagner Date: 4/19/01

Study Director: Judith B. DeJoseph Date: 4/22/01

MicroBioTest, Inc.